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Pathogen Prevalence and Influence of Composted Dairy Manure Application on Antimicrobial Resistance Profiles of Commensal Soil Bacteria

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Abstract

Composting manure, if done properly, should kill pathogenic bacteria such as *Salmonella* and *Escherichia coli* O157:H7, providing for an environmentally safe product. Over a 3-year period, samples of composted dairy manure, representing 11 composting operations (two to six samples per producer; 100 total samples), were screened for *Salmonella* and *E. coli* O157:H7 and were all culture negative. Nonpathogenic bacteria were cultured from these compost samples that could theoretically facilitate the spread of antimicrobial resistance from the dairy to compost application sites. Therefore, we collected soil samples (three samples per plot; 10 plots/treatment; 90 total samples) from rangeland that received either composted dairy manure (CP), commercial fertilizer (F), or no treatment (control, CON). Two collections were made appoximately 2 and 7 months following treatment application. Soil samples were cultured for *Pseudomonas* and *Enterobacter* and confirmed isolates subjected to antimicrobial susceptibility testing. Three species of *Enterobacter* (*cloacae*, 27 isolates; *aeroginosa*, two isolates; *sakazakii*, one isolate) and two species of *Pseudomonas* (*aeruginosa*, 11 isolates; *putida*, seven isolates) were identified. Five *Enterobacter* isolates were resistant to ampicillin and one isolate was resistant to spectinomycin. All *Pseudomonas* isolates were resistant to ampicillin, ceftiofur, florfenicol, sulphachloropyridazine, sulphadimethoxine, and trimethoprim/sulfamethoxazole and most isolates were resistant to chlortetracycline and spectinomycin. *Pseudomonas* isolates were resistant to an average of 8.6, 7.9, and 8 antibiotics for CON, CP, and F treatments, respectively. No treatment differences were observed in antimicrobial resistance patterns in any of the soil isolates examined. Results reported herein support the use of composted dairy manure as an environmentally friendly soil amendment.

Introduction

Dairy cattle have been identified as an important reservoir of foodborne pathogenic bacteria such as *Salmonella* (Edrington *et al.*, 2004a, 2004b) and *Escherichia coli* O157:H7 (Wells *et al.*, 1991; Chapman *et al.*, 1993) and although the number of dairy farms has decreased, farm size (number of animals per farm) and milk production have increased (USDA-NASS, 2002). Furthermore, dairy-producing regions within the United States tend to be more concentrated than in the past and this increase in animal concentration creates new environmental concerns and challenges.

Manure production and its subsequent disposal has been a continual challenge for the livestock producer. Recent estimates report that

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132 million metric tons of dry manure is produced annually on livestock operations in the United States, which is then applied to approximately 9.2 million hectares of land (USDA-ERS, 2005; USDA-NASS, 2005). Traditionally, manure has been applied to agricultural fields with the primary benefit of providing a disposal route and secondarily, a source of fertilizer for the land. Consequently, such applications result in potentially significant environmental problems and minimal economic benefits. Application of manure to agricultural land has been documented as a major source of ground and surface water contamination (Reddy *et al.*, 1981; Carpenter *et al.*, 1998; Pote *et al.*, 2003). Human cases of *E. coli* O157:H7 infection have been documented that resulted from the consumption of agricultural crops or water that had been fertilized with or contaminated by livestock manure (Ackers *et al.*, 1998; CDC, 1999, 2001). Additionally, the increased size of livestock operations and subsequent manure production often exceeds the "holding" or "disposal" capacity of the available agricultural land.

The composting of manure provides two benefits. First, the composting process should raise and sustain internal temperatures of the manure to sufficiently kill fecal bacteria, such as *E. coli* O157:H7 and *Salmonella*, providing for an environmentally safe product (EPA, 1993; Deluca, 1997; Pell, 1997; Lung *et al.*, 2001). Second, it provides an economic benefit for the livestock producer via the development of an environmentally sound waste management system, for the composter via the sale of the composted manure end product, and for the environment via the reduction of runoff pollution and chemical use, improved efficacy of water use, vegetation establishment, and bioremediation (Mukhtar, 2005; Butler *et al.*, 2006).

Granted, the concept of composting manure is certainly not a new one and numerous compost products have been available for some time, yet not all compost is guaranteed to be pathogen free. A survey of 16 composting facilities reported that approximately one third were not meeting minimum standards for pathogenic bacteria (Soares *et al.*, 1995). Certain factors such as inadequate temperatures, aeration, and C/N ratios can lead to the production of contaminated material. Vegetable plants may become contaminated with pathogenic bacteria prior to harvest following application of improperly composted manure (Solomon *et al.*, 2002). Others have reported that *E. coli* O157:H7 persisted in soils for up to 217 days following application of experimentally inoculated compost (Islam *et al.*, 2004).

Large-scale application of composted manure as a soil amendment and a method of land reclamation is a recent innovation and, while the benefits could be substantial, it is crucial to ensure that such large-scale compost production and land applications are effective, safe, and harmonious with the environment. Therefore, the objective of the current research was to evaluate the composting process and to assess the potential environmental impact of large-scale compost application. Specifically, we examined dairy manure compost samples for *Salmonella* and *E. coli* O157:H7, pathogenic bacteria often found in dairy cattle manure, and evaluated antimicrobial resistance profiles of commensal soil bacteria following the application of dairy manure compost to determine if there was a transfer of resistance elements from the dairy to the application site.

Materials and Methods

Compost sample collection

Dairy manure compost samples were collected in November 2003 from 11 commercial composting operations. Four samples were collected at each operation and combined, and a 40-g subsample was obtained for bacterial culture of *E. coli* O157:H7 and *Salmonella* as described below. A second set of compost samples ($n = 47$) was collected in September 2004, representing eight different composting facilities, following delivery to an experimental application site. Immediately following delivery, two to six compost samples per producer (depending on load size) were collected and mixed (approx. 200 g) and subsampled as above. For the land application experiment conducted in 2005 and described in detail below, compost samples ($n = 9$) from a single commercial compost producer were collected following delivery and immediately prior to land application, as described above.

Manure composting, land application, and soil sampling protocol

Composted dairy manure obtained from a single commercial composting operation was delivered to the experimental site in Central Texas. The site used for this research was located in the Cow House Creek watershed, contained within the larger Leon Creek watershed, and located in the primary training and maneuver areas of the Ft. Hood Military Reservation. Fort Hood is located in the eastern portion of the Edwards Plateau and classified as subtropical, subhumid. Mean annual precipitation averages approximately 310 mm/yr and temperatures average about 35° and 4°C in the summer and winter, respectively. Soil type falls within the NuC-Nuff very stony silty clay loam with a 2–6% slope. The objective of this project was to evaluate the effectiveness of compost as a soil amendment in the restoration of drastically disturbed (such as damage from track and wheeled vehicles due to military training activity) rangeland systems. An experimental design consisting of 30 one-third acre plots was implemented (Fig. 1) and assigned one of three treatments (10 plots/treatment): control (no treatment), commercial fertilizer, or compost. The fertilizer (urea) was applied to provide 91 kg equivalent N/acre and the compost applied at a rate of 15 yd^3/acre to provide approximately 91 kg equivalent N/acre and approximately 45 kg equivalent P/acre. Compost was applied using a rear discharge manure spreader providing a 20-foot swath of compost delivery or 60.5 yd^3 compost/acre. Compost testing for N and P was conducted by the Texas A&M University Soil, Water, and Forage Testing Laboratory. A 10-foot buffer zone of untreated native vegetation was maintained around each experimental plot.

Soil samples were collected from three randomly selected locations (minimum of 1 m between points in each plot) within each treatment plot ($n = 30$ samples per treatment per collection), 2 and 7 months following treatment applications. A soil probe (10-mm diameter) was utilized to collect approximately 20 g of the first 3 to 4 inches of topsoil. Each sample was individually bagged and stored in a cooler for transport to our laboratory for culture of *Pseudomonas* and *Enterobacter* as described below. The soil probe was cleaned and disinfected with two successive applications of an antibacterial solution between each sample collection.

Bacterial culture and isolation

Compost samples were cultured for *E. coli* O157:H7 and *Salmonella* within 8 hours of collection. *Escherichia coli* O157:H7 culture and isolation was conducted using an immunomagnetic separation technique. Briefly, 10 g of compost was enriched in 90 mL of tryptic soy broth for 2 hours at room temperature before incubating for 6 hours at 37°C. Following incubation, 20 μL of anti–*E. coli* O157 antibody-labeled paramagnetic beads (Neogen Corp., Lansing, MI) were added to 1-mL volumes of the above enrichments, mixed, and washed. Fifty microliters of the resulting suspension was spread-plated on CHROMagar™ O157 (DRG International, Mountain Side, NJ) plates (containing novobiocin 10 μg/mL and 2.5 μg/mL potassium tellurite) and incubated overnight (37°C). Pink colonies exhibiting typical *E. coli* O157:H7 morphology were resuspended in phosphate-buffered saline (pH 6.5) and confirmed as *E. coli* O157:H7 using the Reveal® microbial screening test (Neogen Corp.) according to the manufacturer's instructions. *Salmonella* was cultured by enriching 10 g of feces in tetrathionate broth (90 mL, 24 hours, 37°C), followed by a second enrichment (100 μL of the first enrichment in 5 mL of Rappaport-Vassiliadis R10 broth, 24 hours, 42°C), before spread plating on brilliant green agar supplemented with novobiocin (25 μg/mL). Colonies exhibiting typical *Salmonella* morphology were confirmed biochemically using lysine agar and triple sugar iron agar (Difco Laboratories, Detroit, MI).

Soil samples collected from the experimental plots described above were cultured for *Enterobacter* (2-month collection only) and *Pseudomonas* (7-month collection only). Ten grams of each soil sample was enriched in 90 mL of either brilliant green bile broth (*Enterobacter*) or tryptic soy broth (*Pseudomonas*) and incubated (24 hours, 37°C). Following enrichment, each sample was streaked on MacConkey's agar (*Enterobacter*) or *Pseudomonas* isolation agar and

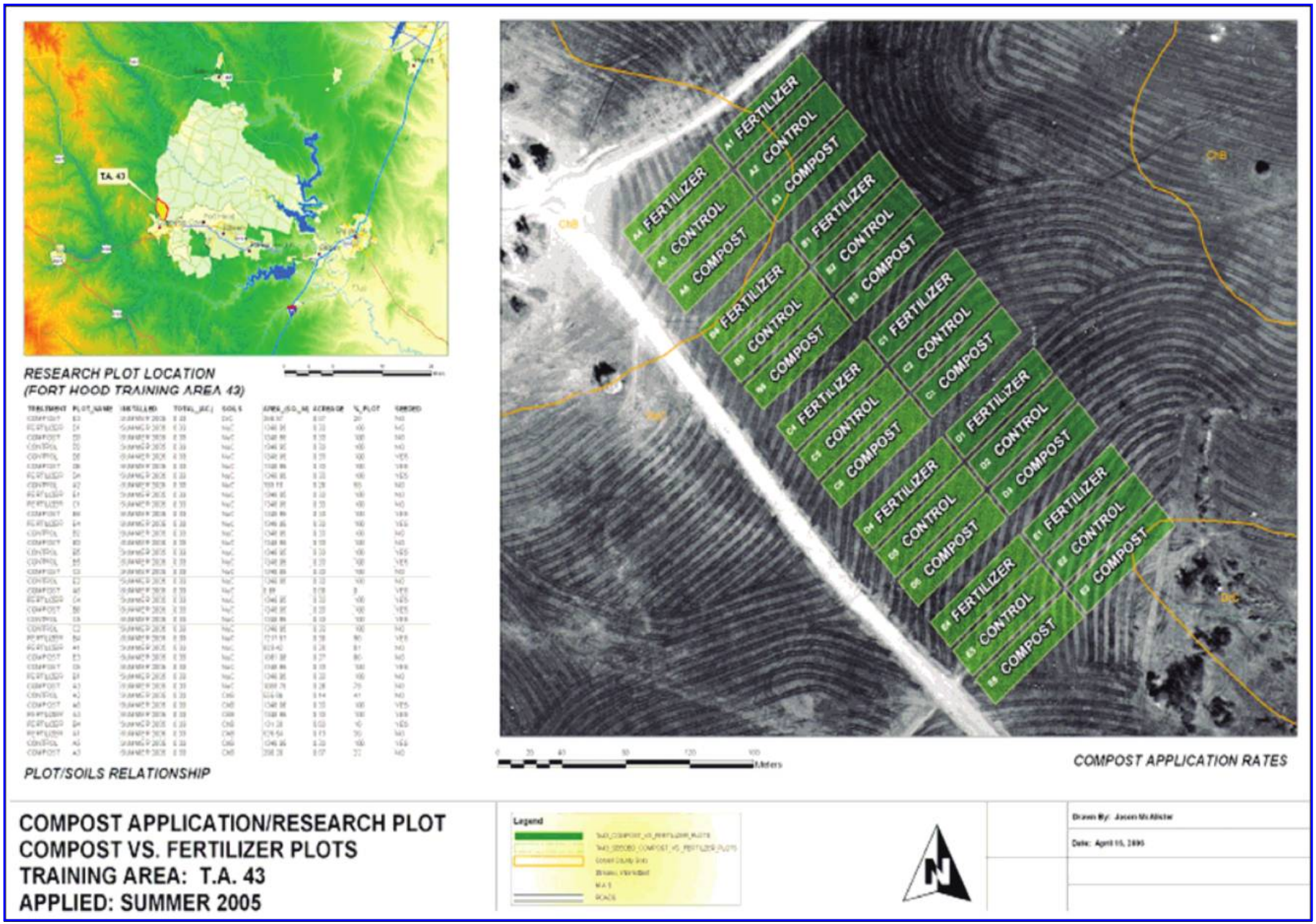

FIG 1. Layout of experimental plots receiving either no treatment (control), commercial fertilizer (91 kg equivalent N/acre), or composted dairy manure (15 yd compost/acre to provide approximately 91 kg equivalent N/acre and 45 kg equivalent P/acre).

incubated for 24 hours at 37°C. Suspect *Enterobacter* colonies were confirmed as *E. cloacae, E. aeroginosa*, or *E. sakazakii* using the API 20E test strip and *Pseudomonas* (*aeruginosa* or *putida*) confirmed using the API 20 NE test strip (bioMérieux, Durham, NC). A portion of the isolates were stored (−80°C) using CryoCare™ bacterial preservers (Key Scientific Products, Round Rock, TX). All media and agar were from Difco Laboratories. Reagents and antibiotics were obtained from Sigma Chemical Co. (St. Louis, MO).

Antimicrobial susceptibility testing

Enterobacter ($n = 30$) and *Pseudomonas* ($n = 18$) isolates cultured from the soil samples collected in 2005 were examined for antimicrobial susceptibility using the Sensititre™ automated antimicrobial susceptibility system according to the manufacturer's directions (Trek Diagnostic Systems, Westlake, OH). Broth microdilution was used according to methods described by the National Committee for Clinical Laboratory Standards (CLSI, 2005) utilizing the National Antibiotic Resistance Monitoring System (NARMS) and bovine/porcine isolate susceptibility testing panels to determine minimum inhibitory concentrations for the following antimicrobials: ampicillin, apramycin, ceftiofur, chlorotetracycline, clindamycin, enrofloxacin, erythromycin, florfenicol, gentamicin, neomycin, oxytetracycline, penicillin, spectinomycin, sulphachloropyridazine, sulphadimethoxine, sulphathiazole, tiamulin, tilmicosin, trimethoprim/sulfamethoxazole, and tylosin. Resistance breakpoints were determined using the National Committee for Clinical Laboratory Standards interpretive standards (CLSI, 2005) unless unavailable, in which case breakpoints in the United States NARMS 2000 Annual Report (FDA, 2000) were used. *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, and *Enterococcus faecalis* ATCC 29212 were used as quality control organisms.

Results

All compost samples ($n = 67$) collected from 2003 to 2005 were found culture negative for *Salmonella* and *E. coli* O157:H7 (data not shown). We were not able to successfully culture, isolate, and identify *Enterobacter* and *Pseudomonas* spp. from all of the soil samples collected; however, 30 *Enterobacter* (27 *E. cloacae*, one each *E. aeroginosa* and *E. sakazakii*) and 18 *Pseudomonas* (11 *P. aeruoginosa*, seven *P. putida*) isolates were cultured and identified for antimicrobial susceptibility screening. The majority of the *Enterobacter* isolates were susceptible to all of the antimicrobials examined and none of the isolates were multidrug resistant. Five *Enterobacter* isolates were resistant to ampicillin (two each in the control and compost treatments and one in the commercial fertilizer treatment) and one *E. cloacae* isolate in the control treatment was resistant to spectinomycin (data not shown).

In contrast, all of the *Pseudomonas* isolates displayed multidrug resistance to the following antibiotics: ampicillin, ceftiofur, florfenicol, sulphachloropyridazine, sulphadimethoxine, and trimethoprim/sulphamethoxazole. In addition, all but one of the isolates was resistant to spectinomycin and the majority of isolates were resistant to chlortetracycline (67%) and oxytetracycline (56%). No treatment differences in antimicrobial susceptibility patterns were observed in the *Pseudomonas* isolates (Table 1). *Pseudomonas* isolates in the control, compost, and commercial fertilizer treatments were resistant to an average of 8.6, 7.9, and 8.0 antimicrobials, respectively. No differences in antimicrobial resistance were observed when examined by *Pseudomonas* species (Table 2).

Discussion

Concerns continue in the Bosque River watershed surrounding water quality issues for nutrient loadings, and, to a lesser extent, pathogens, while concerns build within the Leon River watershed regarding the increased percentage of bacteria. To an extent, the dairy industry has been called to task on the nutrient issues in the Bosque River watershed due to excess phosphorus entering local waterways and ending up in Lake Waco (McFarland and Hauck, 1999; Hauck 2002). Water quality concerns within this watershed have led to the implementation of total maximum daily load requirements (TNRCC, 2001). Based on water quality issues, several state and federally funded programs have evolved, including the

Table 1. Minimum Inhibitory Concentrations (MIC) of Select Antimicrobials and Percentage of Resistant *Pseudomonas* Isolates (by Species and Combined) Cultured from Soil Samples Collected from Rangeland Treated with Fertilizer, Composted Dairy Manure, or No Treatment (Control)

		P. aeruginosa			P. putida			*All* Pseudomonas		
	MIC	*Control*	*Fertilizer*	*Compost*	*Control*	*Fertilizer*	*Compost*	*Control*	*Fertilizer*	*Compost*
Ampicillin	≥32	100	100	100	100	100	100	100	100	100
Ceftiofur	≥8	100	100	100	100	100	100	100	100	100
Chlortetracycline	≥8	100	100	100	0	0	33	71	71	50
Florfenicol	>8	100	100	100	100	100	100	100	100	100
Oxytetracycline	≥8	100	100	75	0	0	0	71	50	43
Spectinomycin	>64	100	100	100	100	100	67	100	100	86
Sulphachloropyridazine	>256	100	100	100	100	100	100	100	100	100
Sulphadimethoxine	>256	100	100	100	100	100	100	100	100	100
Sulphathiazole	>32	0	0	0	50	0	0	14	0	0
Trimethoprim/ sulphamethoxazole	≥4/76	100	100	100	100	100	100	100	100	100

development of dairy manure composting facilities. While compost facilities have improved waste management by reducing the amount of manure that potentially enters local streams, each compost producer has faced quality and consistency issues with both the raw and finished product.

One solution, land applying composted dairy manure transported from a watershed where nutrients are a concern to a watershed that is nutrient deficient, may provide a mutually beneficial impact for two water quality concerns. Utilization of nutrient-rich composted materials on highly disturbed soils in an adjacent watershed provides a means to reduce nutrient concerns in the producing watershed while potentially enhancing erosion control in a neighboring watershed. However, concerns of potential bacterial introductions to the receiving watershed needed to be addressed before the practice could be readily accepted.

Table 2. Multidrug Resistance Shown by the Average Number of Antimicrobials to Which Isolates Displayed Resistance in *Pseudomonas* Isolates (by Species and Combined) Cultured from Soil Samples Obtained on Rangeland Treated with Fertilizer, Composted Dairy Manure, or No Treatment (Control)

	Treatment		
Pseudomonas	*Control*	*Fertilizer*	*Compost*
Putida	7.5	7	6.7
Aeruginosa	9	9	8.75
Both species	8.6	8	7.9

We sampled finished compost representing 11 different composting facilities in the area, and examined these samples ($n = 100$) for *Salmonella* and *E. coli* O157:H7. All samples were found negative for these pathogens. Bacterial growth was observed during the culture process, indicating that either certain species of bacteria were capable of surviving the composting process or the compost had been recontaminated. If the former and these bacteria originated from the dairy, then it is feasible that they could contain elements of antimicrobial resistance and transfer these to other bacteria upon land application of the compost, thereby facilitating the spread of antimicrobial resistance. Other research concluded that soil bacteria in close contact with manure appear to have an important role in the horizontal spread of multidrug resistance (Agersø and Sandvang, 2005). To determine if this is indeed cause for concern, we sampled 30 plots that received either commercial fertilizer, composted dairy manure, or no treatment (control) and cultured two relatively abundant commensal soil bacteria, *Enterobacter* and *Pseudomonas*. Treatment had no affect on multidrug resistance or on the patterns of antimicrobial resistance. Granted, these two bacteria species represent only a fraction of the native soil bacteria population, however they are relatively simple and inexpensive to culture. Furthermore, *Pseudomonas* is a species that readily acquires antimicrobial resistance and should be a good indicator organism of any environmental changes due to compost application. This is ev-

ident in the current research as all of the *Pseudomonas* isolates, across all treatments, were multidrug resistant. Ideally, it would have been preferable to include other bacterial species as well as culture *Pseudomonas* and *Enterobacter* at both collections. However, as the *Enterobacter* spp. isolates examined from the 2-month collection demonstrated limited antimicrobial resistance, and due to financial constraints, we chose to culture only *Pseudomonas* at the 7-month collection.

The addition of organic matter via the application of composted manure can assist in the rehabilitation of degraded lands and bioremediate impacted systems. Such results promote an ecological balance providing for increased water use efficiency and reduced runoff. The results of the current research support the use of composted dairy manure as an environmentally compatible soil amendment that does not facilitate the spread of pathogenic bacteria or elements of antimicrobial resistance.

Acknowledgment

Mention of trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable.

Disclosure Statement

No competing financial interests exist.

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